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Laser Induced Visual Pigment Conversions in Fly Photoreceptors Measured in vivo*

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Abstract. The photochemical cycle of fly visual pigment was studied in vivo with laser methods. Two pulsed dye lasers were used, one delivering the visual pigment converting flash and the other testing the pigment state after a variable interval. Transmission through the rhabdomeres was measured in the eye of blowfly *Calliphora erythrocephala*. It followed that rhodopsin R490 converts into metarhodopsin M580 via two intermediates, with time constants of 700 ns and 80 μs respectively. In the reverse pathway, i.e. the photoconversion of metarhodopsin into rhodopsin, an intermediate decaying with a time constant of 4 μs was found.

Key words: Fly photoreceptors – Visual pigment – Intermediates

Introduction

The predominant pigment in the fly (R1–6 receptors) can exist in two thermostable states, which are photo-interconvertible: rhodopsin absorbs maximally at 490 nm (R490) and metarhodopsin at 580 nm (M580) (e.g., Hamdorf 1979).

As is known from studies on squid visual pigment, rhodopsin converts to metarhodopsin through a series of intermediates (Shichida et al. 1978). Little is known, however, of the photochemical cycle of fly visual pigment. Kirschfeld et al. (1978) and Stark et al. (1979) reported that the formation of metarhodopsin in fruitfly has a time constant of $\simeq$ 0.1 ms (room temperature). This paper presents more detailed experiments on intermediates performed on blowfly in vivo.

* Based on material presented at the Fifth International Congress of Eye Research, Eindhoven, October 1982

Methods

Preparation and Set-Up

The experiments were carried out on *Calliphora erythrocephala* (wild type) at room temperature (≈ 22° C). Flies were prepared by immobilizing them with wax and slicing away a sliver of chitin from the back of the head, so that a light guide can be inserted and thus antidromic illumination can be applied.

The set-up is schematically shown in Fig. 1. The microspectrophotometer (MSP) in which the flies were mounted was constructed from an Ortholux 2 microscope (Leitz). Orthodromic light is supplied by two light sources: *a)* Laser system 1 consisted of a nitrogen laser NL1 (UV12, Molectron) and a home-made Hänsch-type dye laser DL1 (Hänsch 1972; Lawler et al. 1976). In the dye laser a mirror is used instead of a grating, so that the spectral output is determined by the whole fluorescence spectrum of the dye used. This laser system delivered the visual pigment converting flash to the microspectrophotometer via a shutter, a grey filter (Schott) and a dichroic mirror M2 (Schott, 311 or 314) (Stimulus laser). *b)* A 150 W Xe-lamp, controlled by shutters, grey filters (Schott) and interference filters (606 nm or 467 nm, Schott), was used for shifting the photochemical equilibrium of the visual pigment towards the rhodopsin R490 and metarhodopsin M580 state respectively. The red beam (606 nm) created an almost 100% rhodopsin population, the blue beam (467 nm) a 30% rhodopsin and 70% metarhodopsin content (e.g., Hamdorf 1979). The mirrors M1 and M3 are half-mirrors. The possibility of employing laser system 2 as an orthodromic light source was not used in the present experiments.

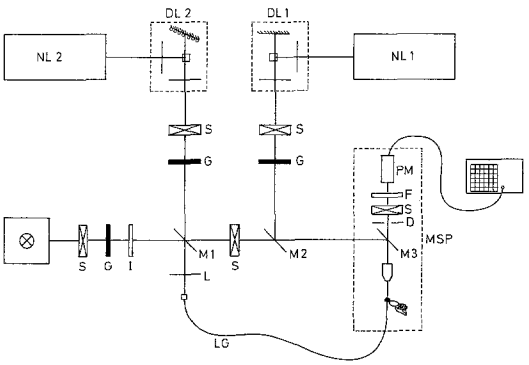

Fig. 1. Experimental set-up. The fly is mounted in the microspectrophotometer MSP. The visual pigment converting flash is delivered orthodromically by laser system 1. The antidromic transmitted light supplied by laser system 2 is measured by a photomultiplier PM and the signal is read from an oscilloscope. The Xe-lamp X provides the continuous adapting light. NL1, NL2, nitrogen lasers; DL1, DL2, dye lasers; S, shutter; G, neutral density (grey) filter; I, interference filter; M1, M2, M3, mirrors; L, lens; LG, light guide; D, iris diaphragm; F, band filter or highpass filter (see text)

Antidromic light is also supplied by two light sources: *a)* The tunable dye-laser DL2 (home-made) pumped by nitrogen laser NL2 (AVCO) composed a second laser system (Test-laser). Influence of this laser on the R-M equilibrium is prevented by adjusting its intensity with neutral density filters. *b)* The Xe-arc lamp. In this case a 586 nm interference filter I (Schott) was used. Both light sources were focussed on light guide LG by lens L. Antidromic transmitted light emerging from the entire deep pseudopupil (Franceschini 1975) is focussed by a UM 20 objective (Leitz) in the plane of a diaphragm D in front of photomultiplier PM (EMI 9862). Shutter S and filter F (585 nm highpass filter, Schott, or K45 band-pass filter, Balzers) were installed to suppress the PM signals due to orthodromic illumination. Pulsed signals were read from an oscilloscope (PM3217, Philips) or a Transient Digitizer (R7912 + 7B92A + 7A19, Tectronics). Continuous signals were recorded on a pen recorder (Servogor).

The pulse width of both lasers was 10 ns. The time resolution of the set-up was 40 ns (due to R.F.-noise emanating from the thyratrons of the N_2-lasers). Two dyes, Coumarine 102 (C102) and rhodamine 6G (R6G) (Molectron dye list 1975) were selected. The fluorescence spectrum of C102 covers the absorption spectrum of *Calliphora* rhodopsin R490. The same applies to R6G and metarhodopsin M580. The stimulus laser was equipped with C102 to start the conversion from R to M, while the test laser was tuned to 586 nm (where the difference in absorption coefficient of M and R is extreme) with R6G in the cuvette. In the experiment concerning the back reaction the dyes were interchanged and the test laser was tuned to 456 nm.

Theory

This section gives a mathematical description of transmission changes in the rhabdomere following excitation of the visual pigment by a short light pulse.

Transmission of test light through a rhabdomere of length ℓ can be described by Lambert-Beer's law. Generally the visual pigment molecules will exist in several spectrally different states. Assuming that the molecules in the various states are distributed homogeneously throughout the rhabdomere (see discussion), the intensity of the transmitted test light at time t is

$$I_\ell(t) = I_0 \exp\left[-C\ell \sum_i \alpha_i f_i(t)\right] \tag{1}$$

where I_0 is the intensity of the light entering the rhabdomere, C is the concentration of the visual pigment molecules, α_i is the molecular absorption coefficient of state i at the test wavelength. $f_i(t)$ is the fraction of molecules in state i at time t.

Suppose conversion of fly rhodopsin to metarhodopsin is characterized by $R \rightsquigarrow B \rightarrow L \rightarrow M$, similar to vertebrates and squid (Yoshizawa and Shichida 1982). The first step is a light-induced reaction and the following steps are

thermal decays. The time development of the fractions f_i in the dark is given by the following set of differential equations:

$$\frac{d}{dt} f_R(t) = 0 \,, \tag{2}$$

$$\frac{d}{dt} f_B(t) = -\tau_B^{-1} f_B(t) \,, \tag{3}$$

$$\frac{d}{dt} f_L(t) = \tau_B^{-1} f_B(t) - \tau_L^{-1} f_L(t) \,, \tag{4}$$

$$\frac{d}{dt} f_M(t) = \tau_L^{-1} f_L(t) \tag{5}$$

where τ_i is the lifetime of state i.

This set can be solved for a given set of initial conditions. A simple case is that where all the molecules are in the rhodopsin state and a short light pulse (short compared to the lifetimes of the individual states) is delivered to the eye converting a certain fraction p of the molecules to the B state. Or, $f_R(0^-) = 1$ and $f_R(0^+) = 1 - p$, $f_B(0^+) = p$ and $f_L(0^+) = f_M(0^+) = 0$. This reduces the general solution to:

$$f_R(t) = 1 - p \,, \tag{6}$$

$$f_B(t) = p \exp\left[-t/\tau_B\right] , \tag{7}$$

$$f_L(t) = p\tau_L(\tau_L - \tau_B)^{-1}\left\{\exp\left[-t/\tau_L\right] - \exp\left[-t/\tau_B\right]\right\} , \tag{8}$$

$$f_M(t) = p(\tau_L - \tau_B)^{-1}\left\{\tau_L(1 - \exp\left[-t/\tau_L\right]) - \tau_B(1 - \exp\left[-t/\tau_B\right])\right\} . \tag{9}$$

When a test wavelength is applied at which the absorption of R can be neglected, i.e., $\alpha_R/p \ll \alpha_M$ ($p = 0.5$ in the present experiments), and assuming α_M and α_L are of the same order of magnitude and $\tau_B \ll \tau_L$, then with (1), (6)–(9):

$$\begin{aligned} I_\ell(t) = I_0 \exp\big[& -pC\ell\left\{\alpha_M + (\alpha_B - \alpha_L)\exp\left[-t/\tau_B\right]\right. \\ & \left. + (\alpha_L - \alpha_M)\exp\left[-t/\tau_L\right]\right\}\big] . \end{aligned} \tag{10}$$

It follows from above that $I_\ell(0^-) = I_0$.

Introducing $T(t) = I_\ell(t)/I_\ell(0^-)$, Eq. (10) can be written as

$$\begin{aligned} \ln\left[T(t)/\mathrm{T}(\infty)\right] = & -pC\ell\left\{(\alpha_L - \alpha_M)\exp\left[-t/\tau_L\right]\right. \\ & \left. - (\alpha_B - \alpha_L)\exp\left[-t/\tau_B\right]\right\} . \end{aligned} \tag{11}$$

For $t \gg \tau_B$ this expression becomes

$$\ln\left[T(t)/T(\infty)\right] = -pC\ell(\alpha_L - \alpha_M)\exp\left[-t/\tau_B\right] . \tag{12}$$

A derivation along the same lines can be given for the conversion from M580 to R490. Here one intermediate state, K, is assumed. The step from M to K is light-induced and the step from K to R is thermally determined. This leads to the following differential equations:

$$\frac{d}{dt} f_M(t) = 0 , \tag{13}$$

$$\frac{d}{dt} f_K(t) = -\tau_K^{-1} f_K(t) , \tag{14}$$

$$\frac{d}{dt} f_R(t) = \tau_K^{-1} f_K(t) . \tag{15}$$

The maximal fraction of molecules in the M state, that can be achieved by adaptation, q, is maximally 0.7 (see above). In such an adaptation condition ($f_M(t) = q$ and $f_R(t) = 1 - q, t < 0$) a light pulse is presented to the pigment with a wavelength as to minimize the excitation of R and leave sufficient excitation of M, so that the conversion of R to M may be neglected. Part of the molecules, a fraction r, will be converted to K. This leads to the following initial conditions: $f_M(0^+) = q - r$, $f_K(0^+) = r$, $f_R(0^+) = 1 - q$, and the solution reads:

$$f_M(t) = q - r , \tag{16}$$

$$f_K(t) = r \exp[-t/\tau_K] , \tag{17}$$

$$f_R(t) = r(1 - \exp[-t/\tau_K]) + (1 - q) . \tag{18}$$

Inserting (16) through (18) into (1) it follows that

$$I_\ell(t) = I_0 \exp\left[-C\ell\{\alpha_M(q - r) + \alpha_R(1 + r - q) + (\alpha_K - \alpha_R) r \exp[-t/\tau_K]\}\right] . \tag{19}$$

From (1) we get $I_\ell(0^-) = I_0 \exp\left[-C\ell\{\alpha_M q + \alpha_R(1 - q)\}\right]$. So

$$T(t) = I_\ell(t)/I_\ell(0^-) = \exp\left[-rC\ell\{\alpha_R - \alpha_M + (\alpha_K - \alpha_R) \exp[-t/\tau_K]\}\right] . \tag{20}$$

This leads finally to

$$\ln[T(t)/T(\infty)] = -rC\ell(\alpha_K - \alpha_R) \exp[-t/\tau_K] . \tag{21}$$

Results

Lifetimes of Intermediates in the R to M Conversion

The intermediate states existing in the rhodopsin → metarhodopsin pathway were studied as follows. First the eye was illuminated with red light $\lambda = 606$ nm to create a nearly 100% rhodopsin content. Subsequently, the antidromic

transmitted light from the test laser with wavelength 586 nm was measured before $[I(0^-)]$ and a variable time interval after the blue stimulus pulse $[I(t)]$. Since the necessarily bright red adaptation light activates the system of migrating pupillary granules the eye was dark-adapted for 1 min prior to the measurements to let the pupil relax (Kirschfeld and Franceschini 1969; Franceschini and Kirschfeld 1976; Stavenga 1975). Now for every measurement transmission is calculated according to

$$T(t) = I(t)/I(0^-).$$

The result is shown in Fig. 2. Each data point represents the average value of eight measurements. The results are explained as follows. Within the time resolution of the set-up some of the R-molecules are transferred by the light flash to a first intermediate state. Subsequently these molecules decay to a second intermediate state, with a lower absorption coefficient (at 586 nm). Finally the molecules decay further to metarhodopsin which in turn has a higher absorption coefficient than the second intermediate. We assume that these states are equivalent to bathorhodopsin and lumirhodopsin as known from the photochemical cycle of squid (Shichida et al. 1978).

The lifetimes of the intermediates can now in principle be calculated from (11) and (12). However, as we measured from the entire deep pseudopupil part of the photomultiplier signal is due to stray light. In order to determine this background a separate experiment was performed. Transmission of continuous test light of 586 nm is measured in the red-adapted (606 nm) and in the blue-adapted (467 nm) state. Comparing the measured value of I (467)/I (606) with the calculated value [from Eq. (1): $I(467)/I(606) = \exp\{-C\alpha_M l f_M(467)\}$, neglecting absorption of R at 586 nm again and using $f_M(467) = 0.7$ and $Cl\alpha_M(586) = 2.7$ in close agreement with the literature (Hamdorf et al. 1973;

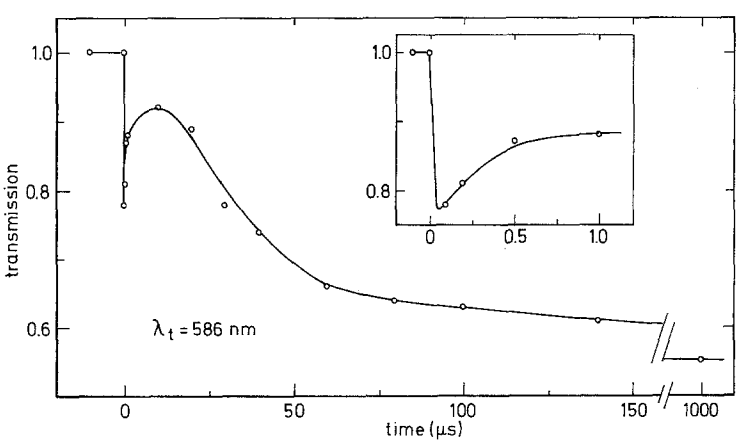

Fig. 2. Antidromic transmission at 586 nm before and after a blue light flash given at $t = 0$. The inset shows the first microsecond. Each measurement of a data point was preceded by a red illumination of 606 nm and a 1-min period in darkness. The standard deviation of the data points is 4–10%

Stavenga 1976; Hamdorf 1979; Schwemer 1979)] yields the background. Further calculations were carried out on background-corrected values of $T(t)$ and $T(\infty)$.

The lifetime of lumirhodopsin is now calculated by fitting the data points with time delays larger than 10 μs to (12) using the least squares method. Data from three flies yielded $\tau_L = 80$ μs.

Now we are able to calculate the first term in Eq. (11). So we can use this formula to calculate the lifetime for bathorhodopsin τ_B. The result is 700 ns.

Lifetime of an Intermediate in the M to R Conversion

The same experiment is performed on the reverse reaction with the following changes in the parameters. The adaptation wavelength is changed to 467 nm. The colour of the stimulus laser is changed to yellow and the test wavelength becomes 456 nm.

The result is shown in Fig. 3. Here the transmission changes can be explained with one intermediate having a higher molecular absorption coefficient at 456 nm than R. We call this intermediate K.

Completely analogous to the previous experiments the background (this time at 456 nm) is determined. With the thus corrected values the lifetime of K can be calculated by fitting the data points to (21) with the least squares method. Data from three flies yielded $\tau_K = 4$ μs. We note here that the calculated time constants are distinctly longer than the values obtained assuming a simple exponential transmission change: for the present experiments by about a factor 2. This is due to the exponentially absorbing rhabdomere.

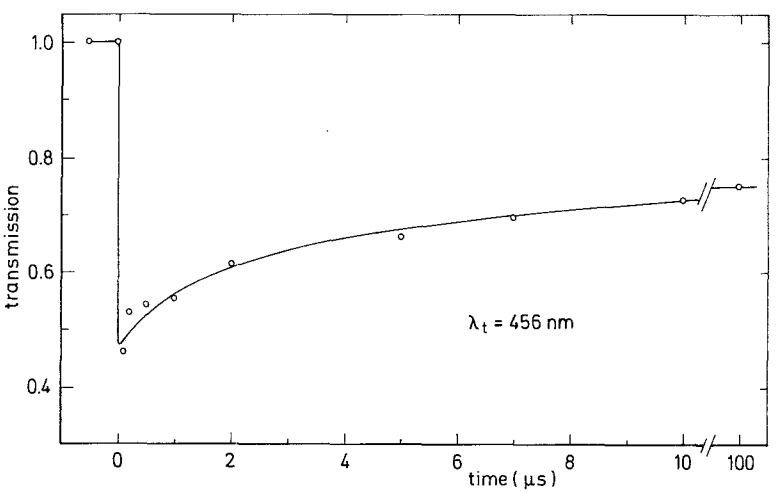

Fig. 3. Antidromic transmission at 456 nm before and after a yellow light flash given at $t = 0$. Each measurement of a data point is preceded by a blue adaptation of 467 nm and 1-min period in darkness. The standard deviation of the data points is 4–10%

Discussion

The obtained experimental results have revealed a number of intermediates in the photochemical cycle of blowfly, schematically presented in Fig. 4. Implicit to this scheme are the assumptions that the excited molecules are distributed homogeneously throughout the rhabdomere and that by the converting flash only the first intermediate is created. Increasing the intensity of the stimulus laser did not convert significantly more visual pigment, indicating that during the flash a photoequilibrium between *R* and *B* (or *M* and *K*) was established. Because in cattle and squid bathorhodopsin *B* can be converted into isorhodopsin, it could be considered that also in fly *B* (or *K*) are photoconverted into another state than *R* (or *M*). However, as isorhodopsin in squid is not formed at temperatures above 0° C (Suzuki et al. 1976) this possibility seems unlikely. Moreover, the initial $[I(0^-)]$ and the final transmission $[I(\infty)]$ measured before and after a stimulus flash respectively remained constant throughout the experiment, indicating that no pigment had disappeared from the *R-M* cycle.

The lifetime of bathorhodopsin measured in frog retina (Cone 1972) and in extracts of squid rhodopsin (Shichida et al. 1978) is 250 ns and 300 ns respectively. These values are comparable to that of blowfly presented here (700 ns). The time constant of the conversion of lumirhodopsin to metarhodopsin (80 μs) appears to be in close correspondence with the value for metarhodopsin build-up in fruitfly reported by Kirschfeld et al. (1978) (125 μs) and Stark et al. (1979) (90 μs). We note that Cone (1972) concluded 70 μs for the lifetime of lumirhodopsin in frog retina.

Kirschfeld et al. (1978) also found that at 0° C rhodopsin is photoregenerated from metarhodopsin within 300 μs. We have discovered an intermediate *K* decaying with a time constant of 4 μs (Fig. 3 and 4), but other work suggests that *K* converts via an intermediate *N* into rhodopsin *R* at a slower rate (Shang and Kirschfeld, personal communication). Since this intermediate was not detected at the test wavelength of 456 nm it is likely that *N* and *R* are absorbing about equally at this wavelength.

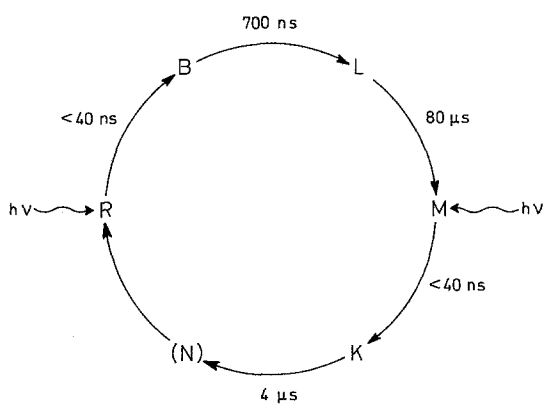

Fig. 4. Schematic representation of the photochemical cycle of blowfly rhodopsin. Rhodopsin *R* excited by light absorption converts to bathorhodopsin *B*. Thermal decay via lumirhodopsin *L* to metarhodopsin *M* follows. The back reaction proceeds via intermediate *K* and possibly *N* (see text). Time constants of the conversion steps are indicated

Obviously further experiments are necessary to clarify details and also to substantiate correspondences and deviations between the photochemical cycle of flies and other related visual pigment systems (squid, Shichida et al. 1978; *Ascalaphus,* Hamdorf et al. 1973; barnacle, Minke et al. 1974; *Limulus*, Ostroy 1977).

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